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Alternative Hypotheses for the Role of Promotion in Chemical Carcinogenesis

by Van Rensselaer Potter*

A new protocol for carcinogenesis in rat liver is described in order that confirmatory experiments might be undertaken concurrently. The basic protocol, designated IPI (initiator + promoter + initiator), is presented in several alternative forms, including the possible use of X-irradiation as the initiator. The rationale is discussed in terms of the two-hit somatic mutation theory of Armitage and Doll, with an initial hit produced by the first dose of initiator and expansion of single cells to sizable clones by promotion thereby increasing the probability of a second hit by the second dose of initiator. The question of relevant mutations was taken up and it was proposed that genes for chalones (C) and for chalone receptors (R) are logical targets for consideration in a two-mutation sequence. Alternative hypotheses pertaining to promoter action were described in terms of possible mechanisms by which nonelectrophilic promoters might simulate a second mutation by increasing or decreasing the levels of a nonchromosomal replicating particle in target cells.

I believe it is worthwhile to discuss rationally derived hypotheses, concepts and beliefs in connection with the cancer problem because I agree with Peyton Rous, who once said, "Beliefs are important, because what men believe determines what men do." I believe that the initiation-promotion model of carcinogenesis is the key to the understanding of the nature of cancer.

When making hypotheses we should endeavor to construct alternative hypotheses and the means to test them (*1*). I offer two alternative hypotheses for the action of the classical promoters, both of which are based on the assumption of a two-hit or multi-hit mutational process.

Promotion as Hyperplasia Due to Blocked Intercellular Communication

Recently it was proposed that the key to the understanding of carcinogenesis in terms of initiation and promotion is the assumption that more than one relevant mutation is required for the production of a promotion-independent cell (*2,3*). It was suggested on the basis of experiments with rat liver that large numbers of stage I single mutant promotion-dependent cells could be produced by a single injection of a low dose of an initiator or a subcarcinogenic dose of a complete carcinogen. These "initiated" stage I cells were assumed to be held in a nonproliferative state by inhibitors (chalones) produced by surrounding adult hepatocytes (*2*).

Administration of a promoter of liver carcinogenesis, e.g., phenobarbital as demonstrated by Peraino et al. (*4*), was assumed to block "intercellular communication" as shown for the phorbol ester promoters by Yotti, Chang and Trosko (*5*) and by Murray and Fitzgerald (*6*) and later confirmed by Newbold and Amos (*7*). Williams (*8*) reported that phenobarbital affected communication between liver cells. Umeda et al. (*9*) found no effect with Chinese hamster cells [but see Trosko et al. (*10*)]. That phenobarbital expands the population of altered cells in rat liver has been established by Pitot et al. (*11*).

Progression through time in the absence of a second dose of initiator was assumed to occur by spontaneous or uncontrolled mutations, with the probability of a second relevant mutation to promoter independence increasing tremendously as the population of single mutant promotion-dependent cells increased under the influence of a promoter (*2,3*).

The term "conversion" was used to represent the experimental production in liver of a second step in the initiation process by a second dose of initiator acting on one or more cells in the expanded popula-

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tion of stage I cells (2). The calculated numbers of hepatomas caused by two single doses of initiator separated in time, with no intervening promotion (one per 1000 rats) was contrasted with the number of hepatomas caused by two single doses of initiator separated in time with promotion intervening to give clones of average size 1000 cells (one hepatoma per rat). The calculations were based on an initiator dose that would give 1000 stage I cells per liver (2) plus the untested assumption that the number of stage I initiated cells might be comparable to the number of γ-glutamyltranspeptidase (GGT) clones.

The suggested experiment will be termed the IPI protocol, indicating an experiment involving two single injections of an initiator separated by an interval of promotion in the rat liver system. Details of alternative IPI protocols for rat liver are being worked out in experiments now in progress.

The purpose of the present essay is to suggest possible variations on the theme and to document a basis for suggesting what the relevant mutations might be, in order to encourage other laboratories to test concurrently IPI protocols of their own or identical design since the experiments will require up to two years to complete. The present commentary is based on experiments involving rat liver, which are not assumed to be completely analogous to experiments with mouse skin (12).

That the IPI protocol may be an idea whose time has come is suggested by the independent publication of the identical idea in June 1981 by Moolgavkar and Knudson (13), who stated "Finally, a regimen in which application of an initiator is followed by several applications of promoter until papillomas appear and then by another application of initiator should yield more carcinomas than is seen in classical initiation-promotion experiments. We are not aware that this experiment has been done. Such an experiment should probably be done with preformed carcinogens because cells in the intermediate stage may have lost the ability of metabolic activation of the initiator." Earlier, in emphasizing the production of a second mutation in the two-stage hypothesis of Armitage and Doll (14), they remarked, "It should be clear by now that our two stages are not those of initiation and promotion. Rather, promotion expands the population of initiated cells, thus increasing the probability of the second event in one of them." The concordance of the two independent proposals, one in terms of liver, and the other in terms of skin, is noteworthy.

It is of historical interest that in a widely quoted paper on human cancer by Armitage and Doll (14), in a purely hypothetical discussion that did not propose the experiment, we find the statement "In the simplest case, in which the subject is exposed on *two separate and unique occasions* to the effect of the two agents, the resulting incidence of cancer will be proportional to $d_2 n_t$ where d_2 is the dose of the second promoting agent, and n_t is the number of "changed" cells present at a time t after exposure to the first initiating agent." (Italics added). It is clear that in referring to "the second promoting agent" the authors did not imply that the second agent was a promoter in the sense of the terminology that is now widely accepted (15). The second unique occasion would have to be exposure to an initiator in the simplest case and they did not exclude that the second agent could be the same as the first. What is noteworthy is that for 23 years no one appears to have reported an experiment stemming from an application of the concept. It appears possible that the experiment, if conceived by anyone, was considered to be technically unlikely to succeed in skin experiments, while the possibilities in liver experiments have only recently become more obvious. The possible relevance to human cancer etiology and to assays for carcinogens should not be overlooked.

Suggested Protocols

The aim of the IPI protocol for rat liver is to carry out the first initiation step with a range of doses that will produce significant numbers of clones of altered cells that can be enumerated on a per liver or per cubic centimeter basis by quantitative stereology (16) using histochemical methods such as the GGT technique to reveal the clones of altered cells. The dose of initiator should be nonnecrogenic, should produce several thousand altered clones per liver and should be empirically established to be in the subcarcinogenic range as a single dose with or without subsequent promotion.

For diethylnitrosamine (DEN), considerable data are available (11), but the protocols are not directly comparable. In preliminary experiments we have given single intraperitoneal injections of 5, 15, and 50 mg DEN/kg body weight to 22-day-old weanling male rats and to 42-day-old rats from the same lot. After 14 days each group was started on phenobarbital and this was maintained for 2, 4 or 6 months. At 4 months there were about 1100 GGT foci/cm^3 in the rats injected with 50 mg DEN at 22 days of age and about half that many in the comparable group injected at 42 days of age (Richards, Campbell, Dast and Potter, unpublished). Accordingly, the IPI protocol with 5, 10, 20 and 40 mg DEN at either age can be recommended, followed by treatment with or without phenobarbital for 4 months, with a second injection of DEN over the same dose range after a 2-week period without phenobarbital and mainte-

nance without phenobarbital for the remaining life span to quantitate hepatoma production.

If the first injection produced promoter-dependent foci and the second injection produced an occasional promoter-independent cell, the quantification of the latter should be made over an extended time span in the absence of promoter in the time period subsequent to the second injection. If the promoter-independent cells are really the result of two relevant mutations it should be possible to obtain a much larger yield of hepatomas if the second injection is given to animals carrying a large number of expanded foci that have resulted from promoter treatment.

Theoretically, there should be an IPI protocol for a dose of an initiator that will give the following results (see Fig. 1): I + P, many foci, no hepatomas; I + P + I, many foci and several hepatomas; 2I + P, two times as many foci but no hepatomas; P + 2I, few small foci, no hepatomas; I + no P + I, few small foci, no hepatomas. It now appears that the appropriate doses of DEN at each injection will be under 10 mg/kg when phenobarbital is used. Thus in the above protocol, if I = 5 mg/kg in a single injection, 2I = 10 mg DEN/kg in a single injection. Where P appears, it signifies promoter treatment only during the time between two injections of I or an identical period.

In our preliminary experiments, rats injected at 22 days of age had not only more foci but they appeared sooner than in the case of rats injected at 42 days of age. We have attributed this to the presence of endogenous promoters, since Moses et al. have found maximal levels of multiplication-stimulating activity (MSA) in rat serum at birth, decreasing to zero by 28 days of age (*17*) and LaBrecque et al. have used weanling rat liver for the isolation of hepatic stimulator substance (HSS) which they report to be absent in adult rat liver (*18*). It is well known that cell proliferation is active in neonatal and weanling rat liver and nearly complete by 42 days.

Peraino et al. (*19*) have used even younger rats in carcinogenesis experiments. They used day-old female rats injected with approximately 15 mg DEN/kg body weight and placed on a diet containing 0.05% phenobarbital at 21 days of age. They reported that 100% of the animals had foci after 4 weeks and 8 weeks on phenobarbital and five of 24 rats had hepatocellular carcinomas at 8 weeks. Further experiments without phenobarbital treatment are in progress (Peraino, personal communication).

On the basis of the striking effects obtained with young rats, we have begun an experiment that eliminates phenobarbital altogether with an injection of 15 mg DEN/kg body weight at 22 days of age and a second injection at 42 days of age relying on endogenous promoters in the period between 22 and 42 days of age for an IPI protocol. Controls include both 15 and 30 mg of DEN/kg body weight as single injections at age 22 days only and at age 42 days only. If this experiment is based on inappropriate assumptions, then an IPI protocol based on the Peraino report (*19*) should be attempted, with smaller amounts of DEN, e.g., 2 or 5 mg/kg at each of two injections, the first at 1 or 10 days of age and the second at 42 days of age, with no phenobarbital at any time assuming endogenous promotion.

As pointed out by Moolgavkar and Knudson (*14*), the IPI protocol might not be productive with initiators like DEN or any that have to be metabolically activated. In that case an agent such as methyl- or ethylnitrosourea might better be used after the appropriate dose range for foci production had been worked out (*20*). However, with foci as small as 10-20 cells in diameter, even if the altered cells are unable to metabolically activate the initiator, surrounding liver cells might carry out the activation sufficiently to affect cells in the foci (*21*).

A strong basis for assuming that active metabolites are available to the stage I cells is the report by Becker (*22*) that a subcarcinogenic single dose of dimethylnitrosamine following a three-cycle noncarcinogenic regimen of *N*-2-fluorenylacetamide produced 12 hepatocellular carcinomas and 40 nodules in nine rats after one year. This experiment is worthy of repetition.

The IPI protocol for rat liver might very well be successful using X-irradiation as the initiator, on the basis of experiments by Cole and Nowell (*23*) who combined radiation and CCl_4 treatment and came very near to suggesting the IPI protocol.

The Importance of Chalones and Chalone Receptors

The suggested protocol is based on evidence that normal adult differentiated tissues produce tissue specific inhibitors of proliferation (chalones). Moreover the inhibition of metabolic cooperation by promoters (*5-10*) can also be taken to mean that promoters may act among other ways by diminishing the ability of normal adult hepatocytes to inhibit the growth of stage I-initiated cells (Fig. 2).

The word *chalone* (from the Greek word, to make slack) was first proposed by Schafer in 1916 (*24*) to effect a contrast with the word hormone, but it was not used in the current sense until 1962 when Bullough (*25*) first advocated its present use. Iverson has recently reviewed the extensive literature on chalones (*26*).

Holley et al. (27) have recently cited 12 examples of the isolation of growth inhibitory factors from a variety of mammalian cells and tissues. "Nevertheless," they remarked, "there are doubts as to the significance of endogenous growth inhibitors because most of the inhibitor preparations have low specific activity. The inhibitors have been difficult to purify, and with some preparations the causes of growth inhibition have, at least in part, turned out to be trivial." In contrast to some earlier reports they reported the isolation of two kidney epithelial cell growth inhibitors that were active in cell cultures at ng/ml concentrations and whose activity was counteracted by epidermal growth factor (EGF) or by calf serum.

At about the same time Wang and his colleagues reported (28) the isolation of an inhibitor for fibroblast proliferation and McMahon and Iype (29) reported the preparation of an inhibitor of hepatocyte proliferation from adult rat liver. The latter factor has now been reported to be isolated as a protein with molecular weight of 26,000 (30). Any relationship to the low density lipoprotein isolated by Leffert and Weinstein (31), to the material isolated from beef liver by Sekas et al. (32), to the mitosis-inhibiting protein (MIP) (33) or to the "chalone hepatique" (34) remains to be explained.

The assumption underlying the present discussion is that chalones and chalone receptors exist and are involved in the carcinogenic process (Fig. 3). "Peptide hormones induce a host of responses in their target tissues through selective interactions with cell-surface receptors" (35-37), and it is reasonable to assume that the peptide chalones interact with receptors also. While the term chalone may be appropriately used in the broadest sense to include biological effectors that are not peptides, recent work (27-30) has implicated proteins in the range of 10,000 to 30,000 molecular weight. [Further citations given by Iverson (26)]. The current emphasis on the polypeptide "transforming growth factors" (TGF's) (38) is based on a flood of discoveries in the area of the peptide hormones and their receptors (35-38), while advances in terms of chalones and the likelihood that chalone receptors exist have been largely ignored. In referring to the "ubiquitous role" of receptors in metabolic regulation Brown and Goldstein (37) note that "specific receptors have now been demonstrated for more than 30 different physiologically important regulatory molecules" in eight different categories including plasma lipoproteins but with no reference to chalones as such.

Research on chalone receptors depends on the availability of pure preparations of appropriate chalones and the development of serum-free growth media. so that the chalones can be tested in cell culture systems in the presence of known concentrations of known growth factors with which they may compete as indicated by Holley et al. (27). The achievement of all three of these requirements appears imminent.

Liver Growth Factors

The development of serum-free growth media for continuous propagation has been accomplished for many cell lines (39) and may be near at hand for hepatocytes. At issue is the nature of the hepatocyte cell line that might be propagated in serum-free medium and the apparent multiplicity of growth regulators and nutrients required. At present it appears that the mass conversion of adult hepatocytes to a population of proliferating cells *in vitro* has never been accomplished. On the other hand several investigators have developed hepatocyte cell lines that can be subcultured for many generations (29,40). Schaeffer (40) in particular has cloned a culture that originated with cells from livers of 3-day old rats and has documented the presence of liver-specific functions and the normal 42 chromosome diploid karyotype. This clone, designated as RL-PR-C, remains nontumorigenic for many population doublings but eventually transforms spontaneously. Both the early and late passage cells appear to be useful for the development of serum-free culture media, with the latter expected to require a less complex medium (W. Schaeffer, personal communication).

The complex requirements for the regulation of the growth of hepatocytes have been studied by Leffert since 1972 (41,42). In a recent report citing this experience Leffert and Koch (43) emphasized the roles of insulin, glucagon and epidermal growth factor (EGF) and concluded that perhaps the earliest event in response to the peptides was an increased Na^+ influx which may well be related to the TGF's (43). They recognized that additional factors might be involved, referring to publications covering regulation by "possibly even six blood-borne peptides." The question of endogenous growth factors such as multiplication-stimulating factor (MSA) (17) and hepatic stimulator substance (HSS) (18) produced by fetal or neonatal liver cells has been referred to.

Growth of rat hepatocytes in serum-free media through many passages has so far not been reported although Marceau et al. (45) have studied the response of both neonatal and adult hepatocytes to insulin, dexamethasone, triiodothyronine and EGF in serum-free medium using a fibronectin coated substratum.

It is clear that past studies have frequently exam-

ined extracts for inhibitors in the presence of growth factors and vice versa (*32*). With the development of serum-free media the inhibitors could be studied in the presence of controlled amounts of growth factors and the competition for receptors could be quantified.

The Relevant Mutations

The search for the "relevant" mutations that led to transformation of normal cells to malignant cells has gone on for many years (*23*). Current work has emphasized the polypeptide "transforming growth factors" (TGFs) (*38*) and their activity as kinases that act to phosphorylate tyrosine moieties in other kinases and other proteins (*44*). These discussions have ignored the question of possible interaction between TGF's and chalones despite credible reports that indicate such interaction (*27*). Moreover, different affinities in transformed cells as compared with normal cells have been suggested (*28*).

The hypothesis for two-stage carcinogenesis in terms of chalones and chalone receptors is presented in its simplest form in Figure 1, and this hypothesis forms the basis for the protocol and hypothesis outlined in Figure 2. It should be emphasized that the protocol in no way depends on the hypothesis nor would an increased yield of tumors in the IPI protocol necessarily support the hypothesis. Nevertheless, the IPI protocol could provide a useful basis for testing the hypothesis by means of histochemical tests. According to the hypothesis, normal adult liver cells contain receptors (R) for the liver-specific chalone (C) that they produce. Such a chalone has been isolated by McMahon et al. (*30*) as

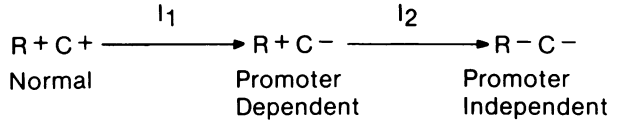

FIGURE 1. Simplified hypothesis for two-stage carcinogenesis. Symbols as in Fig. 2. The first stage is the single mutant cell, which is operationally defined as requiring the presence of a promoter in order to proliferate. The second stage is the double mutant, which is here defined as promoter independent, meaning that it is able to proliferate in the absence of a promoter and in the presence of any chalones naturally present. The two-stage theories of Moolgavkar and Knudson (*13*) and of Armitage and Doll (*14*) also refer to single mutants and double mutants. It is implicit in any single or double relevant mutant hypothesis that additional irrelevant mutations may be present. It is also necessary to point out that mutants in the receptor gene may cover a spectrum between R^- and R^+ with receptors that have varying affinity constants for the matching chalone. The additional stages shown in Figs. 1 and 2 result from the operational distinction between a single mutant cell and a clone of mutant cells (*2*).

well as by others (*26,30-34*). DePaermentier et al. (*34*) used a differentiated hepatoma (that originated in my laboratory) for assay purposes while McMahon et al. (*29,30*) used a "normal" liver cell line similar to that employed by Schaeffer (*40*) and showed that their chalone had no effect on several tumor lines.

From these results it might be inferred that normal adult liver cells are R^+C^+ in phenotype, while some hepatomas are R^+C^- in genotype and have the receptor but do not produce sufficient chalone, and others are R^-C^- in genotype and neither possess sufficient receptor nor produce sufficient chalone.* If an R^+C^- cell is situated in the midst of a population of normal R^+C^+ hepatocytes it is logical to assume that it would be held in a non-proliferating state by chalone (C) produced by the R^+C^+ hepatocytes, which would also restrict each other.

If a promoter acted to block metabolic cooperation or intercellular communication, the R^+C^+ cells would still be blocked internally, but the R^+C^- cells would no longer be blocked by their neighbors and would proliferate (*1,15*).

The hypothetical R^+C^- promoter-dependent cell is not assumed to be identical with any of the presently known "altered cell" phenotypes such as the GGT-positive or ATPase-negative clones that have been studied. Their number may be greater or smaller than the numbers of the other types following initiation and promotion. It seems logical that if the population of single mutant R^+C^- cells can be expanded in the presence of promoter, the possibility of a second mutation yielding the promoter-independent R^-C^- stage will be greatly enhanced. Histochemical immunofluorescence tests based on these two phenotypes might reveal the earliest numbers of such cells in comparison with the numbers revealed by present techniques. Such tests might be developed using fetal and neonatal liver for controls.

It is possible that the three states proposed in Figure 1 would bear a regulatory relationship to the

* The studies on the low density lipoprotein (LDL) receptor by Brown and Goldstein (*37*) provide evidence that the genes for the normal or mutant receptors in their studies are allelic (*46*), that there is a half-normal number of LDL receptors in heterozygotes, and that there is a gene dosage effect (*37,46*). That a critical level of gene products in a heterozygote cell might determine the proliferative rate has been proposed by Trosko and Chang (*3*) and is here emphasized in connection with proposed relevant gene products R (for chalone receptor) and C (for chalone production). Thus when R^+ is used it will indicate the homozygous state R +/+ and when R^- is used it is intended to mean the heterozygote +/−. Similarly C^+ is the homozygote +/+ and C^- is the heterozygote +/−. The homozygotes for R^- and for C^- may well exist in some progressed tumors but they are not essential for the present hypothesis.

presence and activity of the transforming growth factors (TGF's) as suggested below.

Promotion as the Dilution or Amplification of a Nonchromosomal Genetic Element

The two-hit hypothesis of carcinogenesis has been widely assumed to imply two mutations in terms of altered DNA structure, and the "simplest hypothesis" (2) for the role of promoters assumes that their effect is achieved by expanding the populations of one-hit cells thereby making a second hit (alteration in DNA structure) more probable, as outlined in Figures 2 and 3. This assumption is fortified by the knowledge that in general the initiators and "complete" carcinogens are electrophiles that alter DNA structure, while the typical tumor promoters are not electrophiles and do not form adducts with DNA moieties (47). In the opening section, promotion as hyperplasia due to blocked intercellular communication (i.e., without alteration in DNA structure) was presented. Without discarding that hypothesis, we must now consider the alternatives by which a nonelectrophilic promoter could act to produce the effect of a second mutation without acting to alter DNA structure.

Two alternative hypotheses must be considered. They are not mutually exclusive nor are they incompatible with the simplest hypothesis. The first is based on recent experiments and proposals by Varshavsky (48,49) in which the powerful skin tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA) was shown to dramatically increase the incidence of methotrexate-resistant 3T6 mouse cells in culture (49). It was proposed that the result was due to the production by TPA of "locus-unspecific" extra rounds of DNA replication in many different chromosomal domains, of which the production of greatly increased gene copies for dihydrofolate re-

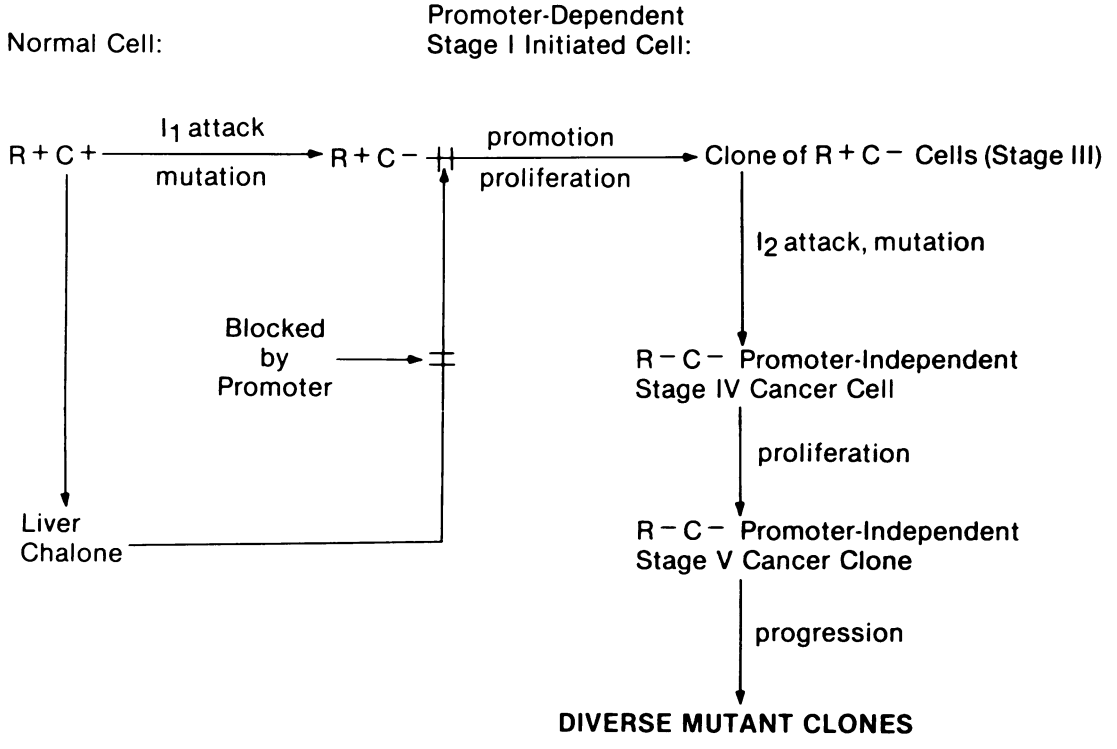

FIGURE 2. The IPI protocol with a hypothesis as to the initiator and promoter functions that would fit the fact of their synergistic action. The symbols R^+C^+, R^+C^-, and R^-C^- represent the normal genome, the single allelic mutant for the chalone, and the mutant for both the chalone receptor (R) and the liver chalone (C). The liver chalone is defined as a product of adult hepatocytes that inhibits the proliferation of mature or immature hepatocytes. The general hypothesis is that "Cancer results from two or more relevant mutations; promoters enhance proliferation of cells with one relevant mutation, thereby increasing the probability of obtaining a cell with two relevant mutations" (2). The specific hypothesis is that mutations in R and C are relevant, here proposed for the first time. I_1 indicates a single injection of a compound that can be classified as an initiator and that produces structural changes in DNA. I_2 indicates a second treatment by the same or another initiator, also as a single injection. Experiments using radiation in two doses separated by an interval of promotion might be ideal.

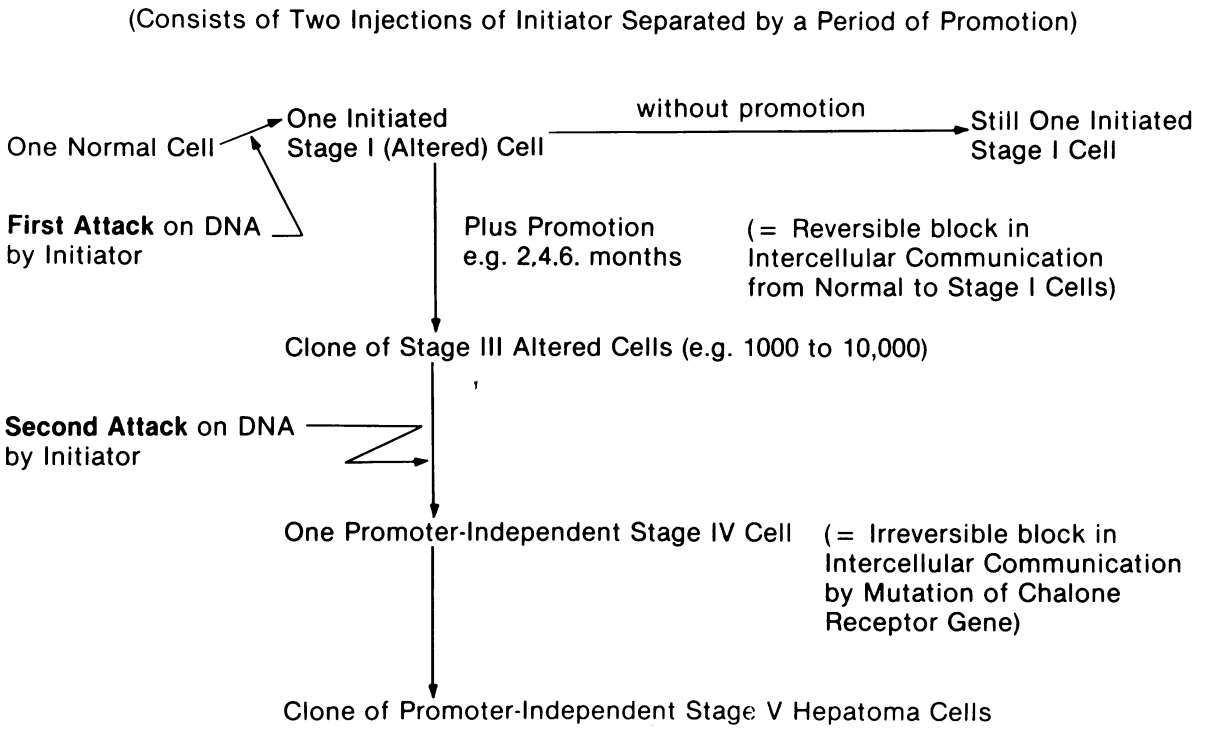

FIGURE 3. A new protocol for chemical carcinogenesis in rat liver. The protocol will be referred to as the IPI since it consists of two injections of an initiator separated by a period of promotion (*2*). The stages are as described earlier (*2*). Stage II is omitted here, since operationally stage II is the point at which promoter is discontinued to determine how many clones are at stage III.

ductase (DHFR) was simply a convenient model. The ability to prevent inappropriate DNA replication leading to the generation of "extrachromosomal copies of cellular genes" (*49*) was assumed to be under metabolic control, in other words it might be said to be controlled by intercellular communication. The "constitutive" production of growth factors by tumors (*38,50*) might, according to the Varshavsky model, be brought about by inappropriate gene amplification in the presence of a promoter, or by a "second mutation" needed for "promoter independence" as in Figures 2 and 3.

A second hypothesis for the production of a relevant "second mutation" by a nonelectrophile tumor promoter resembles the Varshavsky model insofar as it assumes the existence of extrachromosomal copies of genetic particles. It was pointed out in 1950 (*51*) that if such elements exist, their total loss (a "second mutation") could be brought about by a speeding up of cell proliferation relative to particle proliferation or by a slowing of particle proliferation relative to cell proliferation. The latter could be the extra property of promoters in addition to the production of hyperplasia. The 1950 proposal was based on a model system studied by Sonneborn and by his student Preer. I have used tables by Preer (*52*) to construct a chart (Fig. 4) showing the total loss of a nonchromosomal replicative particle under conditions that permit cell replication to exceed particle replication assuming a random (Poisson) distribution of particles between two daughter cells. If a lost particle were the source of control for growth factor production, the latter would become constitutive (*38,50*), and the effect would be that of a second mutation.

The Blocked Ontogeny Hypothesis and the Morris Hepatomas

In an earlier report (*53*) it was suggested that the TGFs are fundamentally the product of embryonic cells or stem cells, in any case cells that actively proliferate, while chalones are the products of nonproliferative cells that are either terminally differentiated or near that stage. It was further proposed on the basis of numerous proteins present as so-called fetal and adult forms of homologous proteins such as γ-globin (fetal) and β-globin (adult) that the production of chalones might somehow not only suppress proliferation but suppress the production of TGF's as suggested above.

Thus a concept of hepatocyte maturation can be presented as in Figure 5 in which the hepatocyte genotype is R^+C^+ but the two genes are unex-

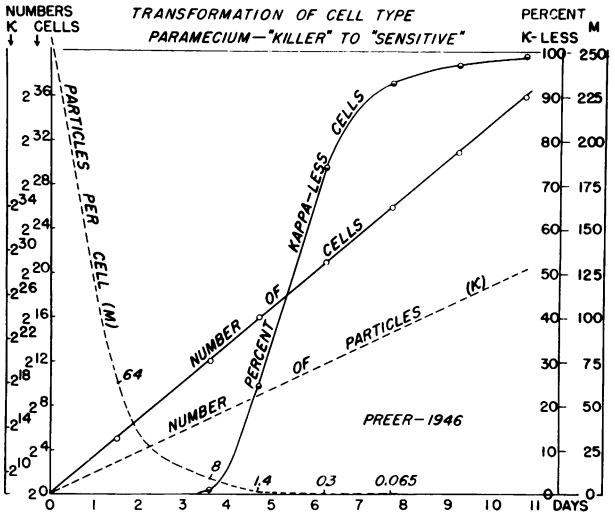

FIGURE 4. An experimental model of the absolute loss of a replicating particle from cells that can divide relatively faster than the particles are able to divide. The model is an alternative hypothesis for a possible "second mutation" by a nonelectrophilic tumor promoter that could slow the replication of a nonchromosomal replicative particle. The experiments were carried out by Preer (*52*), whose data were used to prepare the above chart. See also Potter et al. (*51*). The organism was *Paramecium aurelia* (Variety 2). Preer estimated the starting number of "killer" particles (K. kappa) at 256 (M) and the growth rate as logarithmic at 1.9 times per day for particles and 3.3 times per day for the cells. Distribution of particles in dividing cells was assumed to be random and was calculated according to the Poisson equation $P_0 = e^{-m}$, where P_0 is the number of cells with no particles, and m is the mean number of particles per cell. In the chart, the dashed lines were calculated. The identity and function of the Kappa particles is not relevant to the model as such.

pressed in the fetal hepatocyte, which is accordingly R^-C^- in phenotype. Unstated in Figure 5 is the strong possibility that the fetal hepatocyte is GF^+ in phenotype as suggested earlier (*53*). It is further proposed that the phenotype changes to R^+C^- and finally to R^+C^+ as maturation proceeds, and that in the case of liver these phenotypic changes are reversible in order to facilitate liver regeneration after partial hepatectomy.

Finally, in Figure 6 it is proposed that the spectrum of Morris hepatomas includes some that are R^+C^- and some that are R^-C^-. The former would require a single mutation and the latter would require two mutations. The R^+C^- type would be highly differentiated, subject to some degree of control by the host, and have receptors responsive to chalones (*34*) and as another example, to glucagon, which we have shown (*54*). The R^-C^- type would be poorly differentiated, rapidly growing, and unresponsive to the liver chalone (*29,30*).

The designations R^+ and R^- in Figures 1-5 repre-

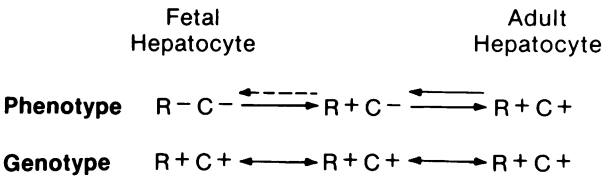

FIGURE 5. The developmental stages of normal hepatocytes. Symbols as in Fig. 2. There are many differences between fetal hepatocytes and adult hepatocytes and it is here assumed that the former do not express either the R or the C gene, while the latter express both. It is also assumed that at least one intermediate stage occurs and that the R^+C^+ phenotype can retrodifferentiate to the earlier forms during liver regeneration, and then undergo "reontogeny."

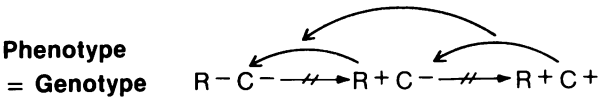

FIGURE 6. Blocked ontogeny in Morris hepatomas. Symbols as in Fig. 2. The curved arrows represent mutational alterations in either or both the R and C genes. Depending on the mutations there could be blocked or partially blocked ontogeny (*53*) at either the R^-C^- or the R^+C^- stage.

sent in the simplest case cells that are either positive or negative but it must be anticipated that there will be a spectrum of affinity constants that will include receptors that are not R^- but that have a weak affinity for the normal chalone. Moreover, the production of chalone will not be a constant but may even vary on a diurnal basis as is the case for the hepatic stimulatory substance (*18*).

Conclusion

As stated earlier, the advocacy of the IPI protocol is not dependent upon the concepts described. What needs to be emphasized is the benefits that will accrue from the cultivation of normal and transformed hepatocytes in serum-free culture in the presence of known concentrations of pure growth factors and chalones. Today the amino acid sequence is known only for EGF (*55*), but the need is for the sequences of all the peptide growth and transforming factors and for the peptide chalones in order to detect homologies, which must form the basis of interaction and competition (*53*). With serum-free cultures (*39*) and pure GFs and chalones (*27*), the receptors and their mechanism of action (*37,46*) can be studied. These studies at the molecular level are needed to finally understand the role of initiation and promotion in carcinogenesis.

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